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Kinetics of the release of the mitochondrial inhibitor protein. Correlation with synthesis and hydrolysis of ATP

Giovanna Lippe [a], M. Catia Sorgato [a] and David A. Harris [b]

[a] *Institute of Biochemistry and CNR Unit for the Study of Mitochondrial Physiology, University of Padova, Padova (Italy)* and [b] *Department of Biochemistry, University of Oxford, Oxford (U.K.)*

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(1) The kinetics of the release of the mitochondrial inhibitor protein (IF_1) is studied in bovine heart submitochondrial vesicles supplemented with ^{125}I-labelled IF_1, using a method for rapidly 'freezing' the state of F_1–IF_1 interaction. It is shown that generation of a protonmotive force leads to release of IF_1 from F_1 into solution, following an exponential process. (2) In one set of experiments the rate of IF_1 release, in IF_1 supplemented vesicles generating a protonmotive force, is correlated with the induction of ATP hydrolytic capacity. It is found that, even under different metabolic states (phosphorylating and non-phosphorylating conditions), both processes follow the same time-course (half-time of around 40 s) and that there is a direct correlation between induced ATPase capacity and IF_1 released. This finding rules out the possibility of a non-inhibitory binding site for IF_1 on the membrane. (3) In a second set of experiments, also using IF_1 supplemented vesicles, the induction of the ATP hydrolytic capacity after energisation is correlated with the induction of the ATP synthetic capacity. Initial rates of both processes are monitored using firefly luciferase, keeping the assay systems as similar as possible. It is shown that the induction of each capacity follows an exponential time-course, with a half-time of around 40 s. This is in good agreement with the half-times obtained for the induction of ATP hydrolytic capacity and the rate of IF_1 release, using the quench-stop method. (4) If the induction of ATP hydrolytic and synthetic capacities is followed in untreated submitochondrial vesicles, i.e., vesicles not supplemented with IF_1, the extent and time-course of the change in both hydrolytic and synthetic capacities remain correlated, but the half-time of the transient falls to around 10 s. It is suggested that the length of the transient, observed in IF_1 supplemented vesicles, results from partial loss of coupling during repeated centrifugations. (5) These results demonstrate that energy-dependent release of IF_1 from F_1 into solution results in a concomitant increase in both ATP synthetic and hydrolytic capacities of the ATP synthase complex, and that the time-course of this process is sensitive to the degree of coupling of the vesicles.

Abbreviations: IF_1, naturally occurring inhibitor protein of mitochondrial ATPase; [^{125}I]IF_1, radioiodinated inhibitor protein; IF_1-SMP, submitochondrial vesicles supplemented with IF_1; [^{125}I]IF_1 SMP, submitochondrial vesicles supplemented with [^{125}I]IF_1; S vesicles, non-phosphorylating vesicles prepared in the presence of 2 mM EDTA (pH 9.2 with ammonia) and passed through a Sephadex G50 column; Ap_5A, P^1,P^5-di(adenosine-5′-)pentaphosphate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; Δp, protonmotive force.

Correspondence: M.C. Sorgato, Istituto di Chimica Biologica, Via F. Marzolo 3, I-35131 Padova, Italy.

Introduction

In recent years, it has become increasingly evident that the activity of ATP synthase in mitochondria is governed not only by the magnitude of the protonmotive force [1–3] but also by a kinetic regulator protein. This protein, commonly known as the ATPase inhibitor protein (IF_1), is a small protein which is found associated with the

F_1-ATPase (ATP synthase) in mitochondria [26] (for reviews, see Refs. 4,5). When F_1 is bound to IF_1, in a 1 : 1 molar ratio, its hydrolytic activity is abolished; conversely, removal of IF_1 from F_1 (for example, when preparing F_1 from mitochondria) leads to an increased total ATPase capacity [5,6].

It has been suggested that also ATP synthesis, by membrane-bound F_1, is inhibited by IF_1 [7,8] (but see Refs. 9–11). However, this effect is short lived. While IF_1-rich ox-heart submitochondrial vesicles have a greatly depressed initial rate of ADP phosphorylation, within approx. 2 min they attain the high rates characteristic of IF_1-deficient vesicles [8,12]. It has been proposed therefore that the IF_1–F_1 interaction is modulated by the 'energy state' of the vesicles such that at high 'energy levels' the inhibitory effect of IF_1 is abolished. In this regard, it has also been shown that generation of a protonmotive force (Δp) in submitochondrial vesicles leads to displacement of IF_1 from F_1 [9,13] and to an increase in ATP hydrolytic capacity [14,15].

Therefore, the simplest model relating ATP hydrolytic capacity, IF_1 content and ATP synthesis rate, in submitochondrial vesicles, might run thus. Generation of a protonmotive force in mitochondrial inner membranes leads (by a mechanism presently unknown) to release of IF_1 from membrane bound F_1 into solution, which results in a concomitant increase in both ATP synthetic and hydrolytic capacities of the assembly of membrane-bound F_1 molecules [7,8,12,13].

This view has been criticised on two grounds. Schwerzmann and Pedersen [9], studying the induction of ATPase activity in energised liver submitochondrial vesicles, conclude that IF_1 release is correlated with the induction of ATP hydrolytic capacity, but that the induction of ATP synthetic capacity is too fast to be correlated with either. Dreyfus et al. [16], in contrast, measure IF_1 remaining on submitochondrial vesicles after energisation and conclude that ATPase induction is not correlated with IF_1 release into solution but with displacement of IF_1 to another, non-inhibitory, binding site.

Both of these views have been challenged on experimental grounds [17], but in view of their incompatibility, both with each other and with the model above, the problem is re-investigated here. In this paper, we present a method for rapidly 'freezing' the state of the F_1–IF_1 interaction in submitochondrial vesicles, which, combined with the use of ^{125}I-labelled IF_1 [13], allows the measurement of the time-course of IF_1 release in the 10 s–10 min range.

It is shown here that the kinetics of IF_1 release are compatible with this release limiting the induction of both ATP hydrolytic and synthetic capacities of the inverted inner membranes of heart mitochondria. All three processes exhibit half-times of around 10–60 s, depending on the state of coupling of the vesicles used. We also show that this correlation is independent of whether a protonmotive force is generated in the presence (phosphorylating conditions) or absence of exogenous ADP. This rules out the possibility that one alternative model or another may hold depending on the metabolic state of the vesicles. We therefore conclude that the simple model, where IF_1 release into solution, ATP hydrolytic capacity and induction of ATP synthesis represent different aspects of a common regulatory mechanism, is sufficient to account for the behaviour of the F_1-IF_1 system in submitochondrial vesicles and hence, presumably, in mitochondria.

Materials and Methods

Mitochondrial preparations. IF_1 (inhibitory activity about 10000 U/mg) was prepared from bovine heart mitochondria as described [6]. Submitochondrial vesicles, low in IF_1, were prepared from Type II bovine heart mitochondria by sonication in the presence of MgATP [18] and suspended in 0.25 M sucrose, 10 mM magnesium acetate. These vesicles gave phosphorylation rates of about 700 nmol/min per mg total protein in buffer A (see below) and ATPase activities of 2–3 μmol/min per mg of total protein, at 30 °C.

Submitochondrial vesicles were enriched in IF_1, according to Ref. 13, except that the resultant vesicles were washed four times by centrifugation through 250 mM sucrose, 10 mM magnesium acetate, 1 mg/ml serum albumin at 4 °C and finally resuspended in this medium. The ratio of IF_1 to membrane protein during the supplementation was 5 μg per mg. The resultant vesicles gave phosphorylation rates of about 300 nmol/min per

mg total protein and ATPase activities of 0.3–1 μmol/min per mg total protein, at 30 °C. Vesicles to be energised by succinate oxidation were further incubated at 37 °C for 30 min in the resuspension medium containing additionally 2.5 mM sodium malonate. This treatment maximally stimulates succinate dehydrogenase activity [19].

Where indicated, [^{125}I]IF_1 (prepared as in Ref. 13) was used for IF_1 supplementation, at a specific radioactivity of about 20000 cpm/μg IF_1. IF_1 was modified to levels of less than 0.5 mol ^{125}I per mol and, as demonstrated previously, labelling affected neither the binding constant nor the energy linked responses of IF_1 [13,17].

Measurement of IF_1 release by rapid centrifugation technique. To measure the dissociation rate of IF_1 and the ATPase activity, submitochondrial vesicles (0.5–1 mg/ml) supplemented with [^{125}I]IF_1, were incubated under continuous stirring in buffer A (20 mM glucose, 10 mM phosphoric acid, 5 mM magnesium acetate, 90 μM Ap_5A, 1 mg per ml bovine serum albumin, 21 mM Tris (pH 7.3)) for 5 min in a perspex cell, thermostatically controlled at 37 °C. Oxidation was initiated by addition of sodium succinate (pH 7.3) to a concentration of 50 mM and oxygenation of the solution maintained by blowing a stream of oxygen gas over the liquid surface. Dissolved oxygen was monitored continuously using a Clark-type oxygen electrode (Rank Bros. Bottisham, Cambridge, U.K.). Where indicated, 180 μM sodium ADP and 25 units of yeast hexokinase/ml were added just prior to succinate addition. The total volume of these additions was such that the final volume of the suspension was increased by 8%. Succinate and not NADH was the substrate chosen because, at the high concentration of vesicles used and over the relatively long periods of time of these experiments, NADH oxidation would have led to an undesired change in pH of the medium. The ethanol-alcohol dehydrogenase regenerating system of NADH was also discarded because it produces acetaldehyde which is potentially toxic to the membranes [20].

Aliquots (150 μl) were removed at intervals and added to 30 μl 'quenching medium', containing 10 mM phosphoric acid, 21 mM Tris (pH 7.3), 45 mM EDTA (disodium salt), 60 μM FCCP (in ethanol), 1.8 mg/ml horse heart cytochrome *c*, and the mixture was cooled on ice. The final ethanol concentration was 0.3% (v/v). 140 μl of this suspension was subjected to centrifugation on a Beckman airfuge (148000 × *g*, 5 min) at room temperature and 100 μl of the supernatant was counted for ^{125}I.

20 μl of the remaining suspension (corresponding to approx. 20 μg protein) were then assayed for ATPase activity, spectrophotometrically, at 340 nm and 30 °C, using an ATP regenerating system [21], in 1 ml of a medium composed of 10 mM phosphoric acid, 21 mM Tris (pH 7.3), 5 mM Mg acetate, 5 mM potassium sulphate, rotenone (2 μg/ml) and 5 μM FCCP. However, since this system would have responded to hexokinase plus glucose in addition to ATPase activity, glucose was routinely removed from the suspension, prior to assay, by preincubation of 40 μl suspension with 100 units of glucose oxidase for 30 min at 37 °C, with constant shaking in vessels open to the atmosphere. Control experiments, run with aliquots not containing hexokinase, showed that changes in ATPase activity of submitochondrial vesicles during such an incubation were negligible within experimental error. However, in order to maintain always the same protocol, the samples with no hexokinase were also incubated with glucose oxidase.

Rate of ATP synthase activation. To measure the induction of ATP synthetic capacity, submitochondrial vesicles (0.08 mg/ml) were incubated under continuous stirring at 30 °C in 0.3 ml of buffer B (21 mM Tris (brought to pH 7.3 with HCl), 5 mM potassium phosphate, 5 mM magnesium acetate, 2 mM sodium AMP) containing 30 μl luciferin/luciferase mixture (LKB ATP monitoring reagent 1243-102) in the thermostatted sample compartment of a luminometer (LKB-Wallac 1251). After 5 min, oxidation was initiated by addition of NADH (final concentration, 1 mM) from an automatic dispenser. After varying periods of oxidation, sodium ADP (100 μM) and potassium phosphate (5 mM) were added from a second dispenser and the increasing light output, corresponding to ATP synthesised, was monitored over the next 20 s. Because of the sensitivity of this technique, dilute suspensions of submitochondrial vesicles can be used and thus oxygenation, over the 2.5 min (maximum) period

of NADH oxidation, is not required.

Control experiments, in which ATP was injected directly into luciferin/luciferase solutions, indicated that the final mixture became uniform only 2–3 s following injection. The first measurement of ATP concentration was made, thus, 4 s after injection of ADP (Table I) and the rate of ATP synthesis measured from the slope of a plot of ATP concentration vs. time over the period 4–20 s, except in the case of simultaneous addition of ADP and NADH, where the slope between 12–20 s was taken due to a departure from linearity at earlier times (see Table I and Results section). Since energisation of the membranes is complete within the 4 s mixing time [8], we expect the rate of ATP synthesis measured in this manner to represent the capacity for ATP synthesis (i.e., the number of F_1 molecules active in synthesis) at the time of measurement (see Results).

To measure the induction of ATP hydrolytic capacity under similar conditions, ADP and phosphate in the second dispenser were replaced by a mixture of sodium ATP and FCCP (final concentrations, 2 μM and 5 μM, respectively) and the fall in light output monitored. In this case, ATP concentration must be kept low to avoid overloading the light detection system and this clearly results in ATPase activity being monitored far below its K_m. However, since rates of enzyme-catalysed reactions are proportional to enzyme concentration at any substrate concentration, relative changes in the rate of ATP hydrolysis measured in this way accurately reflect relative changes in the number of active F_1 molecules.

Membrane protein was determined according to Ref. 22 and IF_1 concentrations according to Ref. 23.

Materials. FCCP was from Fluka AG (Switzerland). Ap_5A (as lithium salt) was purchased from Sigma. Glucose oxidase (from *Aspergillus niger*, grade II, lyophilized) was from Boehringer (Mannheim, F.R.G.). Luciferin/luciferase mixture was purchased from LKB (ATP monitoring reagent 1243-102). ^{125}I was from the Radiochemical Centre (Amersham, Bucks., U.K.).

Results

Correlation of IF_1 release with increase in ATP hydrolytic capacity

To follow the time-course of IF_1 release, submitochondrial vesicles were supplemented with [^{125}I]IF_1. After incubation of the supplemented vesicles in buffer A, they were energised with succinate and aliquots withdrawn at intervals for quenching in a medium containing FCCP, EDTA and cytochrome *c*. The time for sampling and mixing was less than 5 s. The uncoupler, FCCP, abolishes the oxidation-linked protonmotive force and hence prevents any further release of IF_1 as a result of energisation of the vesicles. EDTA (in excess over magnesium ions) abolishes ATP hy-

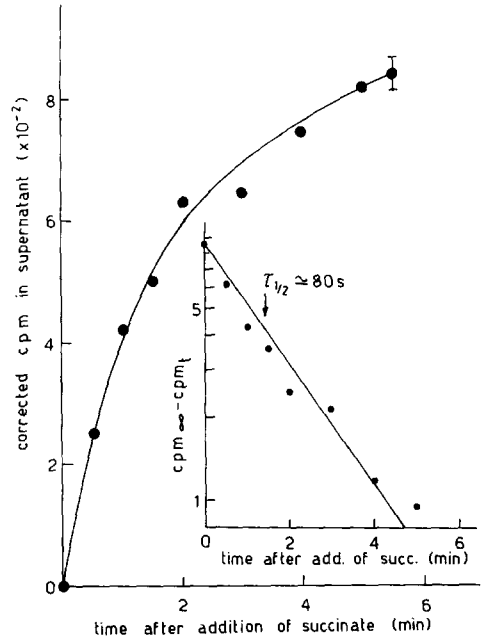

Fig. 1. Time-course of [^{125}I]IF_1 release by [^{125}I]IF_1 SMP upon addition of succinate. At $t = -5$ min, [^{125}I]IF_1 SMP (1 mg/ml) were added to buffer A (see Methods) at 37 °C. Oxidation was initiated by addition of sodium succinate (50 mM) at $t = 0$. Aliquots were removed at intervals for measurement of IF_1 release (see Methods), which was indicated by [^{125}I]IF_1 released to the supernatant. The corrected cpm were calculated by subtracting from the measured values the cpm detected in the supernatant before the addition of succinate. Inset: semilogarithmic plot of Fig. 1. The distance from equilibrium ($cpm_\infty - cpm_t$) is plotted against time after succinate addition. Linearity indicates an exponential change towards an equilibrium value. (cpm_∞) is taken as the value of cpm released after 6 min, after which no change could be observed experimentally.

drolysis and hence prevents rebinding of released IF_1 (which is an ATP hydrolysis dependent process [8]). Cytochrome *c* is present to prevent nonspecific binding of IF_1 (both being positively charged proteins) to the membrane surface.

Fig. 1 shows that this procedure can be used to follow the time-course of IF_1 release from submitochondrial vesicles generating a protonmotive force. The inset shows that this process follows a simple exponential over at least 85% of its time-course, with a half-time of about 1 min. (Over longer periods, a second slower process may be detected [17].) Sampling errors are typically ± 50 cpm (from duplicate measurements), as shown by the bar on the diagram. No IF_1 release is observed if succinate is omitted, or if FCCP (20 μM) is included in the reaction medium (data not shown; see Ref. 13).

It was not possible to correlate ATP synthetic capacity, ATP hydrolytic capacity and IF_1 release in a single experiment because the manual sampling, used to measure IF_1 release, is not sufficiently fast for determining the initial phase of ATP synthesis. On the other hand, sensitive methods of ATP measurements, such as the luciferase assay, do not allow the use of high amounts of particles which are necessary for estimating accurately the release of $[^{125}I]IF_1$. Therefore the approach adopted was to correlate induction of ATP hydrolytic capacity with IF_1 release in one set of experiments and with induction of ATP synthetic capacity in a second set. The two latter processes could then be correlated indirectly through their relation with ATP hydrolytic capacity.

Fig. 2A shows the correlation between the rates of IF_1 release and of the induction of ATP hydrolytic capacity. In these experiments, $[^{125}I]IF_1$ supplemented submitochondrial vesicles were en-

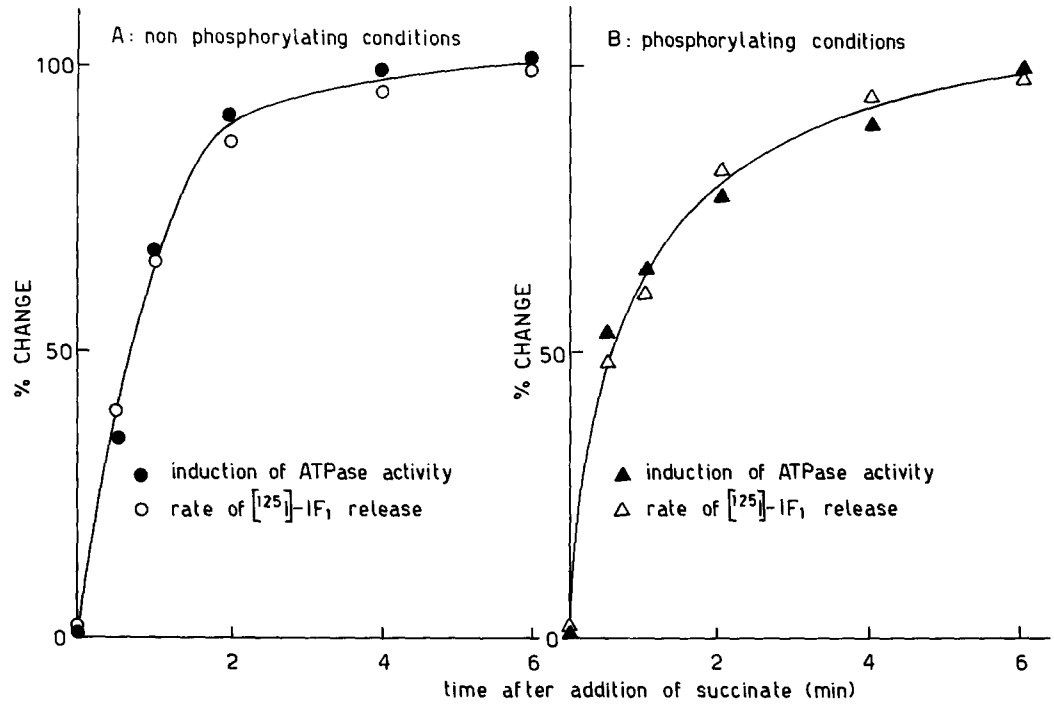

Fig. 2. Correlation between induction of ATPase activity and $[^{125}I]IF_1$ release by $[^{125}I]IF_1$ SMP upon addition of succinate. $[^{125}I]IF_1$ SMP (0.5 mg/ml) were incubated in buffer A (see Methods) and oxidation was initiated as in Fig. 1. Aliquots were removed at intervals for measurement of both ATPase activity and IF_1 release (see Methods). The % change for both parameters was calculated by taking as 100% the difference between the values after 6 min from succinate addition and those found before the addition of succinate. $[^{125}I]IF_1$ cpm were corrected as explained in the legend of Fig. 1. (A) No further addition. (B) Just before succinate addition, hexokinase (25 U/ml) and sodium ADP (180 μM) were added to allow phosphorylation to proceed. ● and ▲, ATPase activity; ○, △, $[^{125}I]IF_1$ release.

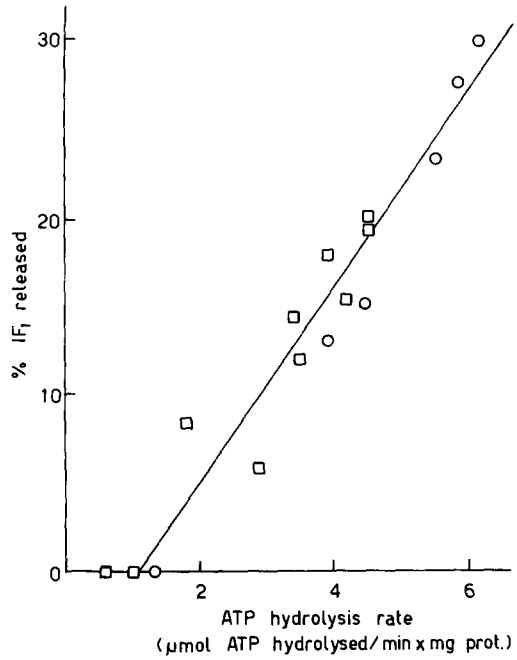

Fig. 3. Relationship between [^{125}I]IF_1 release and ATPase activity by [^{125}I]IF_1 SMP. IF_1 release from oxidizing [^{125}I]IF_1 SMP, after various incubation times, and the corresponding ATPase activities, are taken from the data of Fig. 2. Data of an additional experiment, run as those of Fig. 2A, are also plotted. % IF_1 released is calculated from the ratio between corrected cpm (as in Fig. 1) in supernatant and total cpm in incubation. ○, phosphorylating conditions; □, non-phosphorylating conditions.

ergised by succinate, aliquots quenched in FCCP/EDTA/cytochrome *c*, and the quenched aliquots subdivided for the measurement of ATP hydrolysis (using an enzymic coupled assay system) and for measurement of free IF_1 (by rapid centrifugation). It can be seen that, within experimental error, these two processes can be approximated by a single curve with a half time of about 40 s. Fig. 2B shows that the same correlation holds if ADP and hexokinase are included in the reaction medium, i.e., the vesicles are allowed to carry out continuous phosphorylation.

The direct correlation between induced ATPase capacity and the rate of IF_1 relase can be shown alternatively in the plot of Fig. 3. Data are taken from Fig. 2 and from an additional experiment of the type shown in Fig. 2A. All these experiments were performed on the same submitochondrial preparation. Each time point is here plotted on an ordinate of IF_1 released and on an abscissa of ATPase capacity (i.e., increasing time of succinate oxidation-linked energisation is implicit on moving from left to right). All points, whatever the time of sampling and whatever the state of the particles (phosphorylating or not), lie on a straight line, showing that ATPase capacity is always correlated to IF_1 release.

From Fig. 3 note that (a) the vesicles have a basal ATPase activity of about 1 μmol ATP hydrolysed/min per mg, due to some F_1 molecules being free from IF_1 at the start of the experiment and (b) a loss of about 30% of bound IF_1 leads to an increase of ATPase capacity of (6 − 1 =)5 μmol ATP hydrolysed/min per mg vesicles protein. Thus, by extrapolation, a loss of all IF_1 would lead to vesicles with an ATPase capacity of about 16 μmol ATP hydrolysed/min per mg protein. This is the value predicted from their IF_1 content and the ATPase activity of ammonia-treated 'AS' particles [17], and by direct measurement [27].

Correlation of induction of ATP hydrolytic capacity with induction of ATP synthetic capacity

In this second series of experiments, submitochondrial vesicles were preincubated for varying times with NADH, and then rapidly mixed with either ADP (to measure synthesis of ATP) or with ATP plus FCCP (to measure hydrolysis of ATP). Preincubation and the reaction were carried out in a single cuvette and the reactions monitored using firefly luciferin/luciferase [8]. Readings were taken at 4-s intervals. These data are shown in Table I. We see that the rate of ATP synthesis by these vesicles increases with increasing incubation time to approximately double the initial value after 2 min, showing that more F_1 molecules become available for phosphorylation the longer the incubation with NADH. Table I shows that over 2 min period, ATP hydrolytic capacity also increases about two-fold, with a similar time-course.

The time-course of the changes in the rates of ATP synthesis and hydrolysis over the incubation period with NADH is better shown in Fig. 4. Both inductions follow exponential time-courses (within experimental error), with half-times of approx. 40 s, which is in good agreement with the value obtained for ATP hydrolysis induction and for the rate of IF_1 release, using the quench-stop method

TABLE I

ATP SYNTHESIS AND HYDROLYSIS BY SUBMITOCHONDRIAL VESICLES AFTER PREINCUBATION WITH NADH

IF_1-SMP (0.08 mg/ml) were incubated at 30 °C in buffer B to which had been added luciferase/luciferin reagent and AMP (2 mM) (see Materials and Methods). After 5 min in the chamber of an LKB 1251 luminometer, oxidation was initiated ($t = 0$) by addition of NADH (1 mM) from an automatic dispenser. At the times indicated, phosphorylation was initiated by addition of ADP (100 μM) and phosphate (5 mM), and light emission measured at 4 s intervals for 20 s. The values given in the table represent light output (counts), corresponding to ATP concentration, after each interval of 4 s. The rates of ATP synthesis in parentheses (counts per s) represent the linear rate of increase of light output over 4–20 s, except for the rate corresponding to zero preincubation with NADH, where the rate was linear only between 12–20 s after ADP addition (see Materials and Methods). Induction of ATP hydrolytic capacity was followed under identical conditions except that ADP and phosphate were replaced by 2 μM MgATP and 5 μM FCCP. Decrease in light output was followed for 16 s, and the linear rate of decrease (values in parentheses) calculated over this period. n.d., not determined.

Incubation time with NADH	Light emission (counts) at given times after								
	ADP addition					ATP addition			
	4″	8″	12″	16″	20″	4″	8″	12″	16″
0″	280	310	380	470	565 (24)	1820	1640	1470	1300 (43.5)
15″	360	470	590	700	810 (28)	1780	1550	1320	1100 (56.5)
30″	410	560	710	860	1010 (37.5)	1700	1420	1150	890 (66.5)
45″	420	600	770	950	1120 (44)	n.d.	n.d.	n.d.	n.d.
60″	460	650	840	1030	1210 (47.5)	1700	1380	1080	790 (76)
90″	n.d.	n.d.	n.d.	n.d.	n.d.	1640	1290	980	660 (82)
120″	500	720	930	1140	1340 (52.5)	1590	1210	860	510 (90)

(Fig. 2). Further, the maximal extents of induction can be estimated from the intercept on the y axis of a plot of the reciprocal values of the relative rate vs. the reciprocal values of the preincubation time with NADH (inset, Fig. 4), which is of about 230 for both capacities, as compared to an initial value (without preincubation) of 100. Thus, ATP synthetic and hydrolytic capacities of submitochondrial vesicles appear to increase, following energisation, in parallel. Since we have correlated induction of ATP hydrolytic capacity with both the rate of IF_1 release (previous section) and induction of ATP synthetic capacity (this section), we conclude that all three processes are correlated with each other.

The data of Table I show that the rates of ATP hydrolysis and synthesis are constant over the measuring period (4–20 s after ATP or ADP addition), except where ADP and NADH were added simultaneously. In this case, a constant rate was obtained only after 12 s following addition (see legend to Table I). This period of suboptimal phosphorylation may reflect the build up of membrane energisation; that is, despite ATP synthetic capacity being roughly constant over this time, maximal rates of ATP synthesis cannot occur until Δp has reached its maximal value. (This has occurred by the time of ADP addition in the other measurements, and no such period is observed.) Since we expect the build up of Δp to be rapid in submitochondrial particles [8], this explanation must be speculative; however, it is supported by the data given below.

In all these experiments, the half-times measured for the three processes lie around 40 s (Figs. 2 and 4). This is longer than the time constant previously measured for the initial 'lag phase' in phosphorylation, continuously monitored with luciferase [8,9]. This discrepancy is probably explained by the different degree of coupling of the two preparations. In previous experiments, submitochondrial vesicles were prepared from Type I heart mitochondria [18] and were rich in IF_1 [8]. Hence they were washed by centrifugation only once before use. Here, Type II mitochondria were used [18], unlabelled IF_1 was introduced by direct incubation with submitochondrial vesicles and the vesicles washed four times before use (for direct comparison with the experiments using $[^{125}I]IF_1$). The above proposition is confirmed in Fig. 5, in which the time-course of induction of ATPase activity and ATP synthesis are compared in once

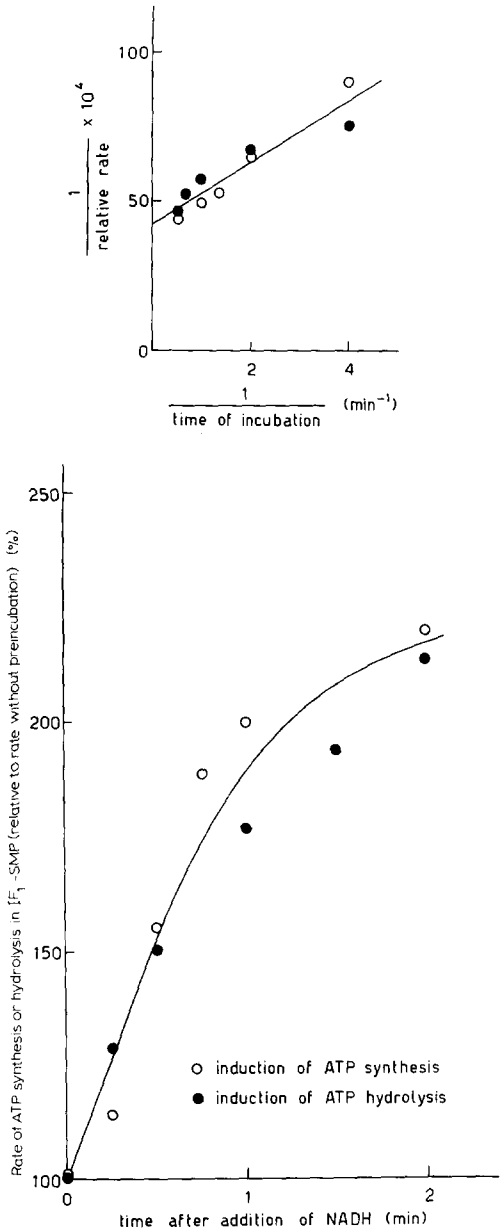

Fig. 4. Time-courses of induction of capacities for ATP synthesis and ATP hydrolysis of IF_1 SMP at different times upon addition of NADH. The rates of ATP synthesis (○) and ATP hydrolysis (●) by IF_1 SMP after varying times of incubation with NADH were calculated from the data of Table I. Rates are plotted here relative to the rates observed when ADP or ATP were added simultaneously with NADH (100%) (see Materials and Methods). Inset: the values of the relative rates of ATP synthesis and hydrolysis at $t = \infty$ were estimated by extrapolation of the plot of the reciprocal values of the relative rate vs. the reciprocal values of the time of incubation (with NADH) to $1/t = 0$. ○, induction of ATP hydrolysis; ●, induction of ATP synthesis.

washed and four times washed IF_1-supplemented submitochondrial vesicles. Clearly, the changes observed in the untreated vesicles are much faster (half-time of 13 s) than the ones that are four times washed and are consistent with the faster time constants previously observed. We found that untreated vesicles were able to generate, upon substrate oxidation in absence of exogenous ADP, a protonmotive force of 170 mV (data not shown, but see following paper), whereas the four times washed particles could sustain a protonmotive force of 145 mV (data not shown). This finding and the lower rate of steady-state phosphorylation maintained by the treated vesicles (see Materials and Methods) argue in favour of a loss of coupling upon prolonged centrifugation. A slowing of induction of phosphorylation with increasing uncoupling has also been shown elsewhere [8,12].

The finding that the washed vesicles are less coupled that the untreated ones also supports the

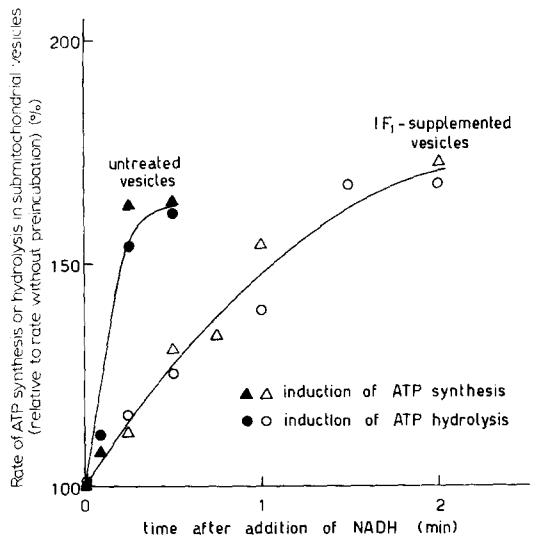

Fig. 5. Time-courses of induction of capacities for ATP synthesis and ATP hydrolysis, measured with luciferin-luciferase assay, of untreated vesicles and IF_1, SMP at different times upon addition of NADH. Untreated submitochondrial vesicles (once washed) (0.08 mg/ml) or IF_1 supplemented vesicles (four times washed) (0.08 mg/ml) were subjected to various times of incubation with NADH, followed by initiation of ATP synthesis or hydrolysis as in Table I, except that phosphate was omitted during preincubation (buffer B minus phosphate, see Materials and Methods). Relative rates were calculated as in Fig. 4. △ and ▲, induction of ATP synthesis; ○ and ●, induction of ATP hydrolysis.

idea that the short period of suboptimal phosphorylation observed with zero preincubation time in the washed vesicles (above, Table I) may be due to slow build up of Δp. Such a period is not observed in the better coupled, untreated vesicles (data not shown).

As mentioned in the legends, the experiments on IF_1-supplemented vesicles of Figs. 4 and 5 were run under the same conditions, except that, during the preincubation with NADH, phosphate was present in the experiments of Fig. 4 and absent in those of Fig. 5, respectively. It is evident that the preincubation in the presence of phosphate (Fig. 4) stimulates the maximal induction of the rates of ATP synthesis and hydrolysis with respect to where it is absent (Fig. 5). Though this stimulating mechanism is as yet unknown, nonetheless it is clear that the similar response elicited in both the rates of synthesis and hydrolysis of ATP by phosphate argues in favour of a same mechanism regulating the two processes.

Discussion

In this paper, we employ rapid quenching and centrifugation procedures to monitor the time-course of IF_1 release from its binding site on F_1 into solution. In IF_1-supplemented submitochondrial vesicles, it is demonstrated that the energy-dependent IF_1 release is a simple exponential process (Fig. 1), which is strictly correlated with the increase in ATP hydrolytic capacity of the mitochondrial inner membranes (Fig. 2). Even under different metabolic conditions, the half-time of the two coupled processes is about 40 s (Fig. 2). (We have previously shown [17] that, over longer periods, a second, 20-fold slower, phase in IF_1 release and increase in ATPase capacity occurs. This slow phase does not appear to be correlated to ATP synthetic capacity and is not considered further here.) This correlation appears to hold over all possible IF_1 contents of mitochondrial membranes; Fig. 3 shows that the extrapolated ATPase activity for vesicles with no IF_1 present is about 16 μmol ATP hydrolysed/min per mg, the value expected from their IF_1 content and the turnover rate of membrane bound F_1 [17].

The release into solution of IF_1, concomitant with an increase in ATPase activity, has been noted previously [9,13] but has been questioned by some workers [16], who proposed a second binding site for IF_1 on F_1. We can find no evidence for such a binding site, even a transitory one. If such a site exists, energy-dependent induction of ATPase capacity would always be greater than IF_1 release and the correlation demonstrated in Figs. 2 and 3 would not exist. As already reported [17], even on the basis of original results of Dreyfus et al. [16], such a secondary binding site for IF_1 would be present at a molar ratio of less than 0.05 per mol F_1.

In the second section, we use the luminometric method to monitor both ATP hydrolysis and synthesis following the generation of a protonmotive force in submitochondrial vesicles. The assay systems, for the determination of the rates of induction of ATP synthetic and hydrolytic capacities on the same preparation of IF_1-supplemented vesicles, were kept as similar as possible to allow the correlation of the time-courses of the two processes. The rate of ATP synthesis observed will depend on both Δp and the amount of active F_1 molecules, viz on both available energy and ATP synthase capacity. The method of measurement was designed to obviate as far as possible the effect of Δp so that synthase capacity alone could be estimated – although in one case, some allowance for the effect of Δp was necessary (see Results).

We find that, following the generation of a protonmotive force in IF_1 supplemented vesicles, ATP synthetic capacity and hydrolytic capacities increase in parallel with a half-time of around 40 s (Fig. 4). In addition, the fractional increase in each capacity converges on a common value at infinite time (inset of Fig. 4), viz both synthetic and hydrolytic capacities increase from 100 to 230 (arbitrary units).

The similarity of these time-courses, and their approach to a similar limiting value, suggest that the same factor is responsible for the energy-dependent change in both capacities. This conclusion is strengthened by the data in Fig. 5, where it is shown that if the experimental conditions are changed such that the time-courses change (e.g., phosphate is omitted from the preincubation buffer or unsupplemented particles are used), the effect on both processes changes in parallel. Since

we have correlated IF_1 release with increasing ATP hydrolytic capacity above, it follows that IF_1 release also limits development of synthetic capacity – a conclusion strengthened by the similarity in half -times (about 40 s) for all three processes in similar preparations (Figs. 2 and 4).

The similarity we observe in the half-times for increases in hydrolytic and synthetic capacities – certainly these times do not vary outside a factor of two from our data (Figs. 4 and 5) – contrasts with the observations of Schwerzmann and Pedersen [9] who concluded that induction of ATP hydrolysis was nearly two orders of magnitude slower than that of synthesis. The reasons for this discrepancy are uncertain; it may be due to different preincubation conditions used in the two measurements by these workers, to the presence of an unresolved second slow phase in the induction of hydrolysis in their experiments (see Ref. 17) or simply to some difference between the rat liver system used in Ref. 9 and the bovine heart system used here.

Another discrepancy between these and earlier results [8,9] is that transients in ATP synthetic capacity have been shown to occur much faster than observed in Fig. 4 here (half-time, 5–10 s as opposed to 40 s). We suggest that the length of the transient observed here depends on the degree of coupling of the vesicles used. In Figs. 1–4 and Table I we show the time-courses of IF_1 release, ATP synthetic and hydrolytic capacities in membrane vesicles supplemented with IF_1 and washed four times by centrifugation. This washing was necessary to decrease background radioactivity when [^{125}I]IF_1 was used to supplement submitochondrial vesicles and was maintained subsequently for ease of comparison. However, this treatment was found to reduce the maximal attainable protonmotive force by these vesicles from about 170 mV to 145 mV (data not shown). When the experiments were repeated with untreated submitochondrial vesicles, the half-time of the transient falls to around 10 s (Fig. 5). However, the extent and time-course of the change in ATP synthetic and hydrolytic capacities clearly remain correlated. In agreement with our earlier work [8] and with Schwerzmann and Pedersen [9], we find that the observed changes in these membranes are virtually over within 30 s, which explains why the latter found no changes in ATP synthetic capacity after longer preincubations.

In conclusion, therefore, we have demonstrated the temporal correlation of energy-dependent changes in ATP hydrolytic capacity with changes in ATP synthetic capacity and changes in IF_1 content of ox-heart submitochondrial vesicles. This correlation holds under different metabolic conditions (phosphorylating, non phosphorylating) and with different membrane preparations (coupled to a greater or lesser extent). The simple model (see Introduction) for control of F_1 activity therefore remains: IF_1, bound to the catalytic subunit of F_1 [24,25], inhibits both synthetic and hydrolytic activities of this enzyme. When the vesicles generate a protonmotive force, IF_1 is displaced into solution (with half-time of the order of seconds) and either process occurs. Whether ATP synthesis or hydrolysis will predominate after IF_1 release depends on the protonmotive force generated by the membranes, and this aspect of regulation is discussed in the accompanying paper (Ref. 28).

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